



Pathogenesis of highly virulent African swine fever virus in domestic pigs exposed via intraoropharyngeal, intranasopharyngeal, and intramuscular inoculation, and by direct contact with infected pigs



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ABSTRACT

To investigate the pathogenesis of African swine fever virus (ASFV), domestic pigs ($n=18$) were challenged with a range (10^2 – 10^6 50% hemadsorbing doses (HAD₅₀)) of the highly virulent ASFV-Malawi strain by inoculation via the intraoropharyngeal (IOP), intranasopharyngeal (INP), or intramuscular (IM) routes. A subsequent contact challenge experiment was performed in which six IOP-inoculated donor pigs were allowed to have direct contact (DC) with six naïve pigs for exposure times that varied from 24 to 72 h. All challenge routes resulted in clinical progression and postmortem lesions similar to those previously described in experimental and natural infection. The onset of clinical signs occurred between 1 and 7 days post inoculation (dpi) and included pyrexia with variable progression to obtundation, hematochezia, melena, moribundity and death with a duration of 4–11 days. Viremia was first detected between 4 and 5 dpi in all inoculation groups whereas ASFV shedding from the nasal cavity and tonsil was first detected at 3–9 dpi. IM and DC were the most consistent modes of infection, with 12/12 (100%) of pigs challenged by these routes becoming infected. Several clinical and virological parameters were significantly different between IM and DC groups indicating dissimilarity between these modes of infection. Amongst the simulated natural routes, INP inoculation resulted in the most consistent progression of disease across the widest range of doses whilst preserving simulation of natural exposure and therefore may provide a superior system for pathogenesis and vaccine efficacy investigation.

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1. Introduction

African swine fever (ASF) is a highly contagious, often fatal, transboundary disease of domestic and wild suids caused by ASF virus (ASFV) (Tulman et al., 2009). It is the sole member of the *Asfarviridae* family and is a large, double stranded DNA virus with a 170–190 kb genome. Due to the lack of a commercial or experimental vaccine or treatment, large economical losses are associated with ASFV outbreaks (Sánchez-Vizcaíno et al., 2012). Such an event occurred in 2007, when ASFV was introduced into the Republic of

Georgia which has subsequently led to spread to Russia, Armenia, Azerbaijan, Ukraine, and most recently to Belarus (WAHID, 2013), with the range of the virus progressively extending west toward Europe (Costard et al., 2013).

In domestic swine, it is generally accepted that under natural conditions, ASFV is primarily transmitted via direct contact with excreted viral particles through nuzzling and/or ingestion (Sánchez-Vizcaíno et al., 2012; Wardley et al., 1983). However, the detailed mechanism of this mode of transmission has yet to be explicitly defined. Arthropod-borne transmission via *Ornithodoros* spp. ticks, which is a relevant means of transmission of ASFV within the wild suid population and from wild suids to domestic swine, is a minor method of transmission between domestic pigs (Arzt et al., 2010; Jori et al., 2013; Penrith, 2009).

An extensive portion of in vivo ASF research has utilized the intramuscular (IM) route of inoculation (Ballester et al., 2010; Carrasco et al., 1996, 1997; Childerstone et al., 1998; Fernández de Marco et al., 2007; Gómez-Villamandos et al., 1995a, 1995b,

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1997a, 1997b, 1998; Oura et al., 1998; Pan and Hess, 1984; Salguero et al., 2004). This method results in highly reproducible clinical disease. However, since IM inoculation does not result in viral interaction with the oral and upper respiratory mucosal surfaces, it bypasses many of the innate defense mechanisms the virus would normally encounter through natural infection. Therefore, it may be a suboptimal inoculation method to examine the early pathogenesis and previremic stages of disease. Similarly, these limitations make IM challenge, an unnatural model for vaccine assessment.

The conventional pathogenesis of ASF has been investigated in vivo using simulated natural routes of inoculation including intranasal (IN) (de Carvalho Ferreira et al., 2012, 2013a; Greig, 1972; Greig and Plowright, 1970; Heuschele, 1967; Plowright et al., 1968), intraoral (IO) (Boulanger et al., 1967; Colgrove et al., 1969), combined oronasal (ON) (Boinas et al., 2004; Hamdy and Dardiri, 1984; McVicar, 1984; Mebus et al., 1978; Mebus and Dardiri, 1979, 1980) and direct contact/aerosol (DC) (de Carvalho Ferreira et al., 2013b; Wilkinson and Donaldson, 1977; Wilkinson et al., 1977, 1981). Within all of the studies noted, the main route of virus detection was through virus isolation and was usually performed only on the blood, serum or a limited number of tissues. Few have focused on virus detection within the secretions (de Carvalho Ferreira et al., 2012; Greig and Plowright, 1970; McVicar, 1984; Wilkinson et al., 1983; Ekue et al., 1989). While these reports have contributed to current understanding of the viral dynamics and pathogenesis of ASF, they fail to capture some specific information which may contribute to develop the next generation of rationally designed countermeasures. Specifically, confirmation of the primary site(s) of infection and elucidation of virus–host interactions during early pathogenesis would facilitate the process of developing and validating effective prophylaxis. Furthermore, variability in methodologies including virus strain, dose, and route of challenge has confounded comparison across studies. The importance of standardizing experimental models including the description and interpretation of clinical and pathologic findings in experimental ASF has recently been emphasized (Galindo-Cardiel et al., 2013). The goal of such standardization is to facilitate comparison of studies across research groups for the purpose of advancing ASF vaccine development whilst avoiding redundancy of research activities.

In the current study, we compared four routes of challenge of domestic pigs with ASFV in order to characterize route- and dose-dependent viral dynamics and the transmission of ASFV via direct contact. This was done to determine how different means of exposure/inoculation alter the pathogenesis of ASFV in swine and to determine which route most closely simulates natural infection with optimum experimental reproducibility.

2. Materials and methods

2.1. Virus

The highly pathogenic ASFV isolate Malawi Lil-20/1 was originally isolated from *Ornithodoros* sp. ticks during a field outbreak in Malawi in 1983 and obtained from L. Dixon (Institute of Animal Health, Pirbright Laboratory, Woking, Surrey, United Kingdom) (Borca et al., 1998). The stock virus was passaged once in primary porcine macrophages derived from heparinized swine blood. The tissue culture supernatant was then stored as 10^8 50% hemadsorbing dose (HAD₅₀) aliquots at -70°C .

The inoculum was prepared by initially diluting stock virus 10-fold in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco; Invitrogen, NY) containing 1% antibiotic/antimycotic (10,000 units/mL of penicillin, 10,000 $\mu\text{g}/\text{mL}$ of streptomycin, and 25 $\mu\text{g}/\text{mL}$ of Fungizone[®]) (Gibco; Invitrogen, NY) and subsequently performing

additional dilution to achieve the desired viral dose in 2 mL of media.

2.2. Animals

Conventional castrated male Yorkshire pigs from a herd that is demonstrated to be free of porcine reproductive and respiratory syndrome virus were used for all experiments. Animals were dewormed and vaccinated for common porcine pathogens including porcine circovirus 2 prior to arrival at our facility. Pigs were approximately 3–4 months old and 22–27 kg upon arrival. All animal procedures were performed following Protocol 225-10-R approved by the Plum Island Animal Disease Center Institutional Animal Care and Use Committee (IACUC), which ensured ethical and humane treatment of experimental animals. The animal experiments were carried out in BSL-3 Ag isolation rooms at Plum Island Animal Disease Center. Animals were kept within the housing facilities 7 days prior to the start of the experiments to allow for acclimation to the new environment. All animals were fed daily and had unlimited access to water.

All animals except for donors in the DC study were allowed to survive until terminal disease. Severely moribund animals were humanely euthanized intravenously with 85.8 mg/kg of sodium pentobarbital. Animals without clinical disease were humanely euthanized at the end of the study as described above.

2.3. Study design

2.3.1. Direct inoculation studies

Experiments were performed to compare three routes of direct inoculation. Eighteen pigs were used in these experiments. Six pigs were assigned to each of the three inoculation route groupings: intramuscular (IM), intranasopharyngeal (INP) and intraoropharyngeal (IOP). Inoculation route groupings were further subdivided into three inoculation dosage groups: low dose 10² HAD₅₀ ($n=2$), mid dose 10⁴ HAD₅₀ ($n=2$) and high dose 10⁶ HAD₅₀ ($n=2$). IM-inoculated pigs received a 2 mL injection of inoculum within the right semimembranosus muscle. Pigs assigned to INP inoculation were sedated with an intramuscular injection of telazol (3 mg/kg), ketamine (8 mg/kg), xylazine (4 mg/kg), placed in sternal recumbency and 2 mL inoculum was instilled through the nares into the nasopharynx via a 15 gauge silicon catheter (Abbott Laboratories, IL). Pigs assigned to IOP inoculation received 2 mL of the inoculum perorally via syringe with an attached 6 in. sterile metal cannula. Animals were sedated, placed in dorsal recumbency and 2 mL of the inoculum was deposited in the ventral portion of the soft palate and palatine tonsil.

2.3.2. Direct contact (DC) transmission studies

Additional experiments were performed to characterize ASFV transmission from IOP-inoculated pigs and viral dynamics in pigs challenged by direct contact. Donor ($n=6$) and naïve pigs ($n=6$) were initially housed in two separate rooms. Donor pigs were inoculated via the intraoropharyngeal method as described above with 2 mL of inoculum at a dose of 10⁵ HAD₅₀ of the same ASFV Malawi Lil-20/1 described above. Upon detection of pyrexia (rectal temperature greater than or equal to 40°C) in at least 4 donor pigs (6 days post inoculation (dpi)), contact pigs were transferred into the donor room and allowed to come into contact with inoculated pigs. Feed was withheld for approximately 4 h to encourage contact between pigs. After 24 h (1 day post contact (dpc)), two contact animals were removed and isolated into a separate room and two donor animals were euthanized to maintain the donor/naïve pig ratio. This process was repeated at 48 and 72 h (2 and 3 dpc). Contact-exposed animals were then monitored for onset and progression of clinical disease.

Table 1
Clinical signs and scoring value for ASF.

Characteristic	Score	
Behavior and mentation	0	Normal, alert, responsive
	1	Mildly obtunded. Slightly reduced liveliness, stands up unassisted, resists restraint or rectal thermometer
	2	Obtunded. Reluctant to stand but will do so when assisted; decreased resistance to restraint or rectal thermometer
	3	Intermittent ataxia, disorientation, can still stand/walk or will not stand/walk even when assisted, still conscious
	4	Moribund. Nonambulatory, unconscious/nonresponsive
Neurologic signs	0	Normal
	2	Unambiguous neurologic signs (e.g. convulsions, seizures)
Defecation	0	Normal to mildly soft stools
	1	Profuse watery diarrhea, ±mild hematochezia or melena
	2	Severe to marked hematochezia/melena
Body temperature ^a	0	38–40 °C
	1	Temperature greater than or equal to 40 °C at any point of study
	2	Temperature greater than or equal to 40 °C for at least 2 subsequent days
	3	Temperature greater than or equal to 41 °C
	4	Temperature less than 38 °C

^a Temperature scores are never reduced.

2.4. Clinical evaluation

Incidence proportion of ASF was calculated for each dose/route combination as [(number of successfully infected pigs for that dose and route) ÷ (all pigs subjected to that dose and route)]. For all studies, clinical evaluations were performed on all animals until death or termination of the experiment. Clinical signs used to characterize progression of ASF are listed in Table 1. These clinical signs were assigned a numerical value based on severity and significance. The sum of the scores of all clinical signs present for each pig was recorded daily.

2.5. Sample collection and processing

Sample collection including whole blood with EDTA, clotted blood for the collection of serum, swab specimens from the nasal cavity and superficial scrapings from the tonsil of the soft palate were performed on the day of inoculation at 0 days post inoculation (dpi) followed by sample collection every other day. Pigs were manually restrained for sample collection. Whole blood and serum were collected via the jugular vein. Tonsil scrapings were collected with a sterile spoon by gently scraping along the surface of the tonsil of the soft palate, exfoliating superficial epithelial cells while making sure to not disrupt the mucosa. Nasal swabs were collected bilaterally using sterile cotton swabs extended just caudal to the alar fold. Following collection, nasal swabs and tonsil scrapings were immediately immersed in 1 mL of DMEM with 5% antibiotic/antimycotic; then, all samples were transferred on ice to the laboratory for processing. All samples were transferred to cryovials and stored at –70 °C until they were analyzed by virus isolation (VI).

Necropsies were performed as soon as possible following euthanasia or natural death. Tissues collected during postmortem examination included: tonsil of the soft palate, lingual tonsil, nasal tonsil, dorsal soft palate (rostral and caudal), dorsal nasopharynx, caudal nasal turbinate, epiglottis, trachea, bronchial mucosa, lung, thymus, liver, spleen, kidney, adrenal gland, thyroid, pancreas, small intestine, large intestine, bone marrow, and lymph nodes including: retropharyngeal, submandibular, hilar, gastrohepatic, renal, inguinal and popliteal. Urine was also taken via direct aspiration from the urinary bladder at the time of necropsy. Tissue samples collected were placed in cryovials as described above and cryomolds, embedded in Optimal Cutting Temperature (OCT) medium (Tissue-Tek O.C.T. compound, Sakura Finetek, CA) and then frozen in liquid nitrogen. Detailed description of tissues from these

animals will appear in a separate manuscript (Howey et al., in progress).

2.6. Virus isolation and titration

Blood, serum, nasal swabs, tonsil scrapings, urine (postmortem only) and tissues from infected pigs were screened for ASFV by virus isolation and titration (VI) on primary porcine blood macrophage cell cultures prepared from defibrinated swine blood as previously described (Zsak et al., 2005). Presence of virus was determined by identification of infected cells by rosette formation (hemadsorption) and titers expressed as doses calculated by the Spearman–Karber method (Mahy and Kangro, 1996).

2.7. Immunomicroscopy

Microscopic localization of the virus in tissues positive for ASFV via VI was performed via immunohistochemistry (IHC). Immunofluorescence (IFA) was used to detect virus in combination with other cellular markers in order to further determine/characterize phenotypes of infected cells. All primary antibodies were extensively tested prior to this study in sections of tissue from ASFV infected or non-infected pigs, using IHC, to ensure specificity.

OCT-embedded tissue samples for IHC and IFA were cryosectioned onto electrostatically charged glass slides and fixed for 10 min in acetone at –20 °C. Slides were blocked for 2 h at 20 °C with Phosphate buffered saline with Tween (PBST) containing 6% mixed serum and 2% powdered non-fat milk. A mouse monoclonal primary antibody 1D9, targeting the ASFV VP30 (Cuesta-Geijo et al., 2012; Galindo et al., 2012) (kindly provided by Javier Dominguez Juncal, Departamento de Biotecnología, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)), was diluted 1:250 in blocking buffer and applied to tissue sections that were then incubated for 20 h at 4 °C.

For IHC, specific anti-ASFV immunoreactivity was detected using a commercial micropolymer alkaline phosphatase detection system (Mach 3 AP kit; Biocare, CA) as per manufacturers' recommendation with an alkaline phosphatase substrate (Vector Red; Vector Laboratories, CA). Slides were counterstained with Gill's hematoxylin and cover slipped using routine methods. A duplicate serial section of each tissue screened was treated with a mouse monoclonal anti-foot and mouth disease virus (FMDV) antibody (10GA4, targeting FMDV O1-Brugge (Arzt et al., 2009)) serving as a negative control.

Table 2

Clinical characteristics of pigs infected with ASFV by different routes and dosages.

Group	No. infected/total no.	Total days survival (\pm SD)	Pyrexia	Clinical signs	
				Days to onset (\pm SD)	Days to onset (\pm SD)
IM					
Overall	6/6	8.5 (\pm 1.8) ^c	5.2 (\pm 1.2) ^c	4.5 (\pm 1.2) ^c	6.3 (\pm 1.4) ^c
10 ² (n=2)	2/2	10.5 (\pm 0.3)	5.5 (\pm 0.7)	5.5 (\pm 0.7)	7.0 (\pm 1.4)
10 ⁴ (n=2)	2/2	8.0 (\pm 1.4)	5.5 (\pm 0.7)	5.0 (\pm 0.0)	6.5 (\pm 2.1)
10 ⁶ (n=2)	2/2	7.0 (\pm 0.0)	4.5 (\pm 2.1)	3.0 (\pm 0.0)	5.5 (\pm 0.7)
INP					
Overall	5/6	10.3 (\pm 1.9) ^c	5.0 (\pm 2.7) ^c	2.6 (\pm 2.5) ^c	6.2 (\pm 2.8) ^c
10 ² (n=2)	1/2	11.0 (\pm 1.4)	7.0 (\pm 0.0) ^a	7.0 (\pm 0.0) ^a	9.0 (\pm 0.0) ^a
10 ⁴ (n=2)	2/2	11.0 (\pm 1.4)	7.0 (\pm 0.0)	1.5 (\pm 0.7)	5.0 (\pm 1.4)
10 ⁶ (n=2)	2/2	9.0 (\pm 2.8)	2.0 (\pm 0.0)	1.5 (\pm 0.7)	6.0 (\pm 4.2)
IOP					
Overall	4/6	10.5 (\pm 2.4) ^c	5.8 (\pm 1.3) ^c	4.3 (\pm 2.4) ^c	4.8 (\pm 4.0) ^c
10 ² (n=2)	0/2	13.0 (\pm 0.0)	—	—	—
10 ⁴ (n=2)	2/2	10.0 (\pm 2.8)	6.5 (\pm 0.7)	3.5 (\pm 3.5)	6.5 (\pm 5.0)
10 ⁶ (n=2)	2/2	8.5 (\pm 0.7)	5.0 (\pm 1.4)	5.0 (\pm 1.4)	3.0 (\pm 0.0)
DC					
Overall	6/6	10.3 (\pm 1.2) ^c	5. (\pm 1.8) ^c	5.0 (\pm 1.9) ^c	6.8 (\pm 2.0) ^c
1 dpc (n=2)	2/2	10.5 (\pm 0.7)	5.0 (\pm 0.0)	4.5 (\pm 0.7)	7.5 (\pm 0.7)
2 dpc (n=2)	2/2	10.5 (\pm 2.1)	3.5 (\pm 2.1)	3.5 (\pm 2.1)	8.0 (\pm 1.4)
3 dpc (n=2)	2/2	10.0 (\pm 1.4)	7.0 (\pm 0.0)	7.0 (\pm 0.0)	5.0 (\pm 2.8)
IOP donor					
Overall	4/6	— ^b	3.6 (\pm 0.6)	4.0 (\pm 1.7)	4.0 (\pm 2.5)

—, not detected; SD, standard deviation.

^a One animal affected within group.^b Survival not calculated due to predetermined euthanasia.^c No statistical difference between group overall values (IM, INP, IOP and DC).

Multichannel IFA was performed similarly as described for IHC, except ASFV antigen detection was performed in combination with primary antibodies with specificity for host cellular markers including pancytokeratin (180059, Zymed, Invitrogen, NY) for epithelium, CD163 and CD172a for macrophages (MCA 2311, and MCA2312GA, respectively AbD Serotec, UK). After incubation of primary antibodies for 20 h at 4 °C, isotype-specific secondary antibodies labeled with fluorescent dyes (AF 350, 488, 594, and 647, AlexaFluor; Molecular Probes Inc., OR) were applied and slides were incubated for 1 h at 37 °C. Images were obtained using a Nikon Eclipse 90i multichannel epi-fluorescent microscope equipped with 350, 488, 594, and 647 excitation filter cubes and digital camera.

2.8. Statistical analyses

All statistical analyses were performed using R statistical program (R core Team, 2013), employing different R packages (explained in detail below). Statistical significance was considered as $p < 0.05$. All data reported herein is not statistically significant unless explicitly stated.

2.8.1. Survival analysis

Duration of survival for each pig was calculated as the total number of days from inoculation/exposure to natural death or euthanasia due to moribundity. Survival functions were calculated for pigs belonging to the IM, INP, IOP and DC groups and compared through a Cox proportional hazard regression model using the package "Survival" for R software (Therneau, 2013). Multiple comparisons were made: (1) between inoculation/infection routes (IM, INP, IOP and DC), (2) within inoculated groups (IM, INP and IOP) comparing type of inoculation route and dose, (3) within each dose (comparing different inoculation routes), (4) within each route (comparing doses), and (5) within the DC pigs (comparing exposure length). Survival was not calculated for donor pigs (IOP donor group) from the contact experiment since these pigs were euthanized at predetermined times regardless of clinical disease.

2.8.2. Clinical signs, viremia and shedding

For all clinical and virological parameters examined, the mean \pm standard deviation (SD) for each infection group/dose combination was calculated using data values collected per individual animal per time point. The effect of the inoculation/infection route used to establish infection was compared across groups (both within the direct inoculation studies and between direct inoculation and contact studies) for the outcome variables present in Tables 2 and 3. These comparisons were made using regression analysis, using a linear model, considering the 4 types of inoculation/infection route as categorical explanatory variables: intramuscular (IM), intranasopharyngeal (INP), intraoropharyngeal (IOP) and direct contact (DC). A linear model was also used to assess the effects of different exposure levels applied in the pigs infected by direct contact, with exposure as explanatory variable (with 3 exposure levels – 1 day, 2 days and 3 days) and variables present in Tables 2 and 3 as outcome. Linear model assumptions were confirmed through visual inspection of the residual plots. The statistical power of these experiments was analyzed retrospectively using the package "pwr" (Champely, 2012). The study did not have enough power to test for possible interactions of type of inoculation route and dose used for inoculation. The power of the analysis using only the explanatory variable type of inoculation/infection route (determining differences between inoculation/infection groups) varied from 0.58 to 0.31, depending on the number of missing values.

3. Results

3.1. Direct inoculation studies

3.1.1. Inoculation and infectivity

Successful infection of individual pigs was determined by the presence of characteristic clinical signs combined with detection of ASFV within the blood components (viremia). In the IM group, all animals regardless of dose were successfully infected (2/2 high dose, 2/2 mid dose, and 2/2 low dose (100% incidence)). In the INP

Table 3

Virologic parameters of pigs infected with ASFV by different routes and dosages.

Group	No. infected/ total no.	Viremia			Shedding			
		Days to onset (±SD)	Duration (±SD)	Maximum titer (±SD) ^{b,c,g}	Days to onset of tonsil shedding (±SD)	Maximum titer (±SD) ^b	Days to onset of nasal shedding (±SD)	Maximum titer (±SD) ^b
IM								
Overall	6/6	4.3 (±1.6) ^e	5.2 (±1.3) ^e	6.5 (±0.6) ^c	8.0 (±1.2) ^e	2.4 (±0.4) ^e	9.0 (±0.0) ^{a,e}	2.1 (±0.0) ^{a,e}
10 ² (n = 2)	2/2	6.0 (±1.4)	5.5 (±0.7)	6.1 (±0.0) ^c	9.0 (±0.0) ^a	2.8 (±0.0) ^a	9.0 (±0.0) ^a	2.1 (±0.0) ^a
10 ⁴ (n = 2)	2/2	4.0 (±1.4)	5.0 (±2.8)	6.4 (±0.9) ^c	8.0 (±1.4)	2.4 (±0.5)	—	—
10 ⁶ (n = 2)	2/2	3.0 (±0.0)	5.0 (±0.0)	6.9 (±0.2) ^c	7.0 (±0.0) ^a	2.1 (±0.0) ^a	—	—
INP								
Overall	5/6	5.4 (±1.1) ^e	5.6 (±1.7) ^e	7.0 (±1.2)	7.5 (±1.7) ^e	3.3 (±1.0) ^e	7.7 (±3.5) ^e	4.2 (±1.7) ^{e,f}
10 ² (n = 2)	1/2	5.0 (±0.0) ^a	6.0 (±0.0) ^a	5.8 (±0.0) ^a	9.0 (±0.0) ^a	2.8 (±0.0) ^a	—	—
10 ⁴ (n = 2)	2/2	5.0 (±1.4)	7.0 (±0.0)	8.3 (±0.0) ^c	6.0 (±0.0)	4.0 (±0.7)	6.0 (±2.8)	5.2 (±0.5)
10 ⁶ (n = 2)	2/2	6.0 (±1.4)	4.0 (±1.4)	6.3 (±0.4)	9.0 (±0.0) ^a	2.3 (±0.0) ^a	11.0 (±0.0) ^a	2.3 (±0.0) ^a
IOP								
Overall	4/6	5.0 (±1.2) ^e	5.3 (±1.7) ^e	8.5 (±0.1)	5.5 (±1.9) ^{e,f}	5.3 (±1.7) ^f	6.5 (±1.0) ^e	5.2 (±1.1) ^f
10 ² (n = 2)	0/2	—	—	—	—	—	—	—
10 ⁴ (n = 2)	2/2	6.0 (±0.0)	5.0 (±2.8)	8.6 (±0.0)	7.0 (±1.4)	4.4 (±0.5)	7.0 (±1.4)	4.7 (±0.2)
10 ⁶ (n = 2)	2/2	4.0 (±0.0)	5.5 (±0.7)	8.4 (±0.2)	4.0 (±0.0)	6.2 (±2.3)	6.0 (±0.0)	5.8 (±1.4)
DC								
Overall	6/6	5.2 (±1.5) ^e	6.2 (±1.7) ^e	8.8 (±0.3)	3.5 (±2.4) ^f	5.2 (±0.8) ^f	7.7 (±0.6) ^e	4.7 (±0.1) ^f
1 dpc (n = 2)	2/2	4.0 (±1.4)	7.5 (±2.1)	8.7 (±0.2)	1.5 (±0.7)	4.7 (±0.5)	8.0 (±0.0)	4.6 (±0.4)
2 dpc (n = 2)	2/2	5.0 (±1.4)	6.5 (±0.7)	8.8 (±0.4)	4.0 (±2.8)	5.8 (±0.0)	7.5 (±0.7)	4.7 (±0.2)
3 dpc (n = 2)	2/2	6.5 (±0.7)	4.5 (±0.7)	8.9 (±0.5)	5.0 (±2.8)	5.2 (±1.2)	7.5 (±0.7)	4.8 (±0.0)
IOP donor								
Overall	4/6	4.8 (±2.2)	— ^d	7.4 (±3.2)	2.8 (±1.5)	4.1 (±1.2)	6.5 (±1.3)	4.6 (±1.3)

—, not detected; SD, standard deviation.

^a One animal affected within group.^b Log₁₀HAD₅₀/mL.^c All titers derived from whole blood except 'c' below (serum derived).^d Duration of viremia not calculated due to predetermined euthanasia.^e Group overall values (IM, INP, IOP and DC) with a common superscript are not significantly different ($p > 0.05$).^f Group overall values (IM, INP, IOP and DC) with a common superscript are not significantly different ($p > 0.05$).^g No statistical differences were calculated for this group.

and IOP groups, incidence was 100% within the mid (2/2) and high dose (2/2) groups; however amongst low dose animals, 1/2 pigs in the INP group and the entire (0/2) IOP group did not become infected (low dose incidence = 50%, 0% respectively).

3.1.2. Survival

The IM-inoculated pigs had the shortest overall survival (mean 8.5 ± 1.8 dpi, $n = 6$) (Table 2, Figs. 1A and 2), however, no statistically significant differences were found between the IM, INP and IOP groups in the Cox proportional hazard analysis. IM-inoculated pigs also had the shortest survival following onset of pyrexia (mean 4.3 ± 1.9 days, $n = 6$). Mean survival after pyrexia was longest within the low dose group (6 ± 0.0 days, $n = 2$) and equal within the mid and high dose groups (3.5 ± 2.1 days, $n = 2$ per group). Amongst the INP-inoculated pigs, overall mean survival was 10.3 ± 1.9 dpi with 5.5 ± 1.8 days survival following the onset of pyrexia. Survival was shortest within the high dose group (9.0 ± 2.8 days, $n = 2$) compared to the mid and low dose groups (11.0 ± 1.4 days, $n = 2$ per group). Overall survival within the intraoropharyngeal group was the longest compared to all other routes with a mean of 10.5 ± 2.4 dpi ($n = 6$). Survival following onset of pyrexia was 4.5 ± 2.1 days; however, the number of days to onset of pyrexia was the longest (5.8 ± 1.3 dpi).

The dose used for inoculation had a significant effect on the pig's survival, with pigs inoculated with the low dose (10^2 HAD₅₀ ASFV) having a significantly longer survival ($p = 0.02$) than pigs inoculated with a higher dose (10^6 HAD₅₀ ASFV). However, when groups were compared within the same dose (i.e. across route), there were no statistical differences between the different inoculation routes.

3.1.3. Clinical signs

For all route-dose combinations, the most consistent and commonly earliest detected clinical sign was pyrexia ranging from 40 to 41.9°C . Diarrhea, hematochezia and melena were common in all groups during the late stages of infection, except the contact experiment donors. Less consistent clinical signs included obtundation, oculonasal discharge, coughing, and dermal hyperemia which were often transient and variable throughout all groups. Terminal stage ASF was characterized by moribundity including a rapid and substantial decrease in body temperature, lack of response to stimulus, inability to stand or ambulate, and occasionally, seizure-like behavior. There were no statistically significant differences ($p > 0.05$) between the IM, INP, IOP and DC routes for total days of survival, days to onset of pyrexia and clinical signs, and maximum clinical score (Table 2).

Amongst the IM-inoculated pigs, the onset of clinical signs was shortest within the high dose group (Table 2, Fig. 2) including pyrexia (4.5 ± 2.1 days) and obtundation (6.0 ± 0.0 days) which occurred in all pigs ($n = 6$). Melena/hematochezia was observed in one animal from the mid and high dose groups, with the shortest onset (5.0 ± 0.0 days) within the high dose. The overall highest mean clinical score was 6.3 ± 1.4 with the highest mean clinical score observed within the low dose group ($n = 2$). The IM-inoculated pigs, had significantly earlier onset of obtundation and onset of melena/hematochezia ($p < 0.05$) in comparison to every group, or in comparison to the DC and INP routes individually.

The lowest overall number of days to the onset of clinical signs was amongst the INP-inoculated pigs (mean 2.6 ± 2.5 dpi, 5/6

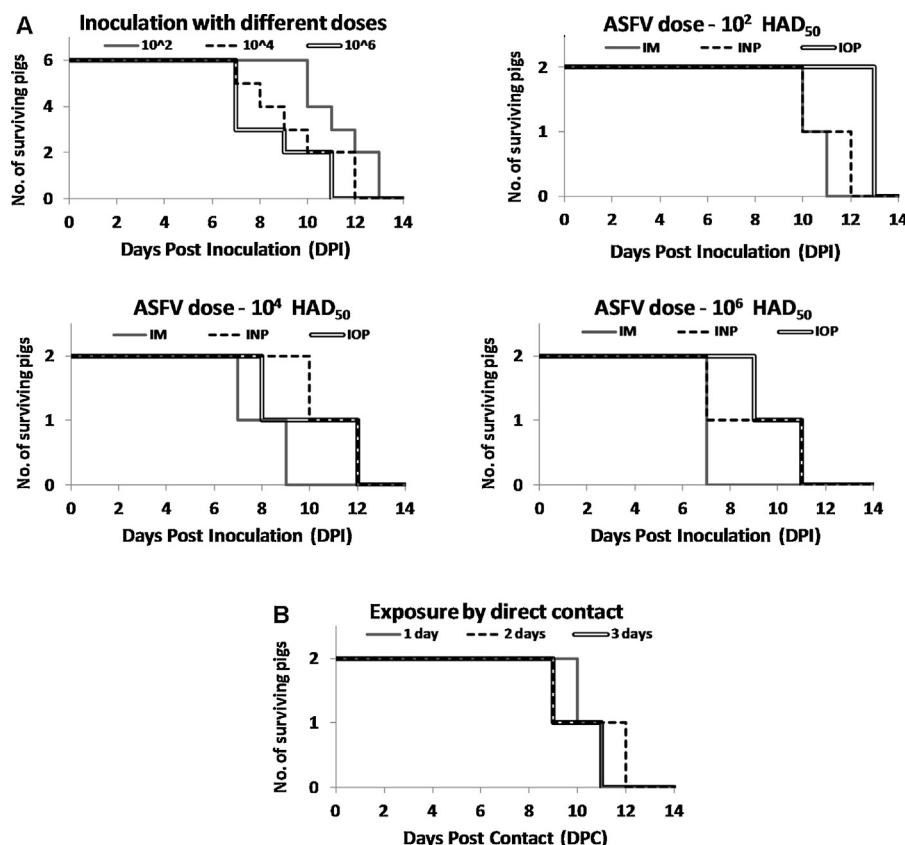


Fig. 1. Descriptive survival analyses for ASFV-exposed pigs relating dose/exposure length to survival duration within the direct inoculation studies (A) and direct contact study (B).

pigs). However, onset within the low dose group was substantially longer (7 days) than the mid and high dose groups (1.5 ± 0.7 days) (Fig. 2D–F). The lowest overall number of days to onset of pyrexia was amongst the INP-inoculated pigs (mean 5.0 ± 2.7 dpi, $n=2$) with the high dose group having the shortest onset (2.0 ± 0.0 days, $n=2$) compared to the low (1/2 pigs) and mid dose (2/2 pigs) groups (7.0 ± 0.0 days). Obtundation and melena/hematochezia occurred in 4 out of 6 pigs with respective means to onset of 9.5 ± 1.0 and 10.0 ± 0.8 days. The mean maximum clinical score amongst the INP-inoculated pigs was 6.2 ± 2.8 with the highest clinical score occurring within the low dose group (9.0 ± 0.0).

The mean maximum clinical score for the IOP groups was 4.8 ± 4.0 (4/6 pigs) with the lowest clinical score found within the high dose group. Clinical signs consistent with ASF were not observed in pigs from the low dose group. Onset of pyrexia occurred within the mid and high dose groups at 6.5 ± 0.7 and 5.0 ± 1.4 days respectively ($n=2$ per group). Obtundation and melena/hematochezia were detected only in one mid dose IOP-inoculated animal starting at 10 and 11 dpi respectively.

3.1.4. Viremia and ASFV shedding

The IM-inoculated pigs ($n=6$) had the shortest overall period to onset of viremia (mean 4.3 ± 1.6 dpi) and number of days of viremia (5.2 ± 1.3 days) (Table 3). Amongst the IM-inoculated pigs, the low dose group had both the longest time before onset and longest duration of viremia (Fig. 2A, Table 3). The highest mean titer of ASFV in serum was detected within the high dose group ($6.9 \pm 0.2 \log_{10}\text{HAD}_{50}/\text{mL}$). Onset to detection of ASFV from the tonsil was shortest within the high dose group. Nasal shedding was only detected in 1 pig within the low dose IM group which occurred at 9 dpi. There was an overall mean of 4.0 ± 1.6 days from onset of viremia to detection of ASFV in secretions. IM-inoculated

pigs had significantly different results in comparison with pigs infected by DC. Specifically these pigs had longer days to onset of tonsil shedding, and lower maximum ASFV titers in tonsil and nasal swabs (Table 3).

The overall number of days to onset of viremia was the longest in the INP groups at 5.4 ± 1.1 dpi (5/6 pigs). The number of days of viremia averaged 5.6 ± 1.7 days with the high dose group having the shortest length of viremia (Fig. 2C, Table 3). The overall mean maximum serum/whole blood titer was $7.0 \pm 1.2 \log_{10}\text{HAD}_{50}/\text{mL}$. Tonsillar ASFV was detected in 4 out of 6 INP-inoculated animals with a mean onset of 7.5 ± 1.7 days and a max titer of $3.3 \pm 1.0 \log_{10}\text{HAD}_{50}/\text{mL}$. Nasal shedding was detected in 3 out of 6 animals with a mean onset of 7.7 ± 3.5 dpi and a max titer of $4.2 \pm 1.7 \log_{10}\text{HAD}_{50}/\text{mL}$. The mean difference between the onset of viremia and onset of ASFV detection in secretions was 1.5 ± 1.9 days. In comparison to DC-exposed pigs, the INP-inoculated pigs had significantly longer days to onset of tonsil shedding (for INP), and significantly lower maximum titers in tonsils.

Within the IOP groups, overall onset of viremia was detected at 5.0 ± 1.2 dpi with overall duration of viremia for 5.3 ± 1.7 days for 4 out of 6 pigs inoculated (Table 3). The high dose group had both the shortest onset to viremia and shortest duration. The mean maximum titer from whole blood was $8.5 \pm 0.1 \log_{10}\text{HAD}_{50}/\text{mL}$. Tonsil and nasal shedding was detected within all infected animals (4/6) with a mean onset of shedding at 5.5 ± 1.9 and 6.5 ± 1.0 dpi respectively. The mean maximum titers for tonsil and nasal swabs for IOP-inoculated pigs were 5.3 ± 1.7 and $5.2 \pm 1.1 \log_{10}\text{HAD}_{50}/\text{mL}$. The period between onset of viremia and viral shedding was the shortest of all routes at 0.5 ± 1.2 days. There were no statistical significant differences in any of the outcome variables between the IOP and the DC groups.

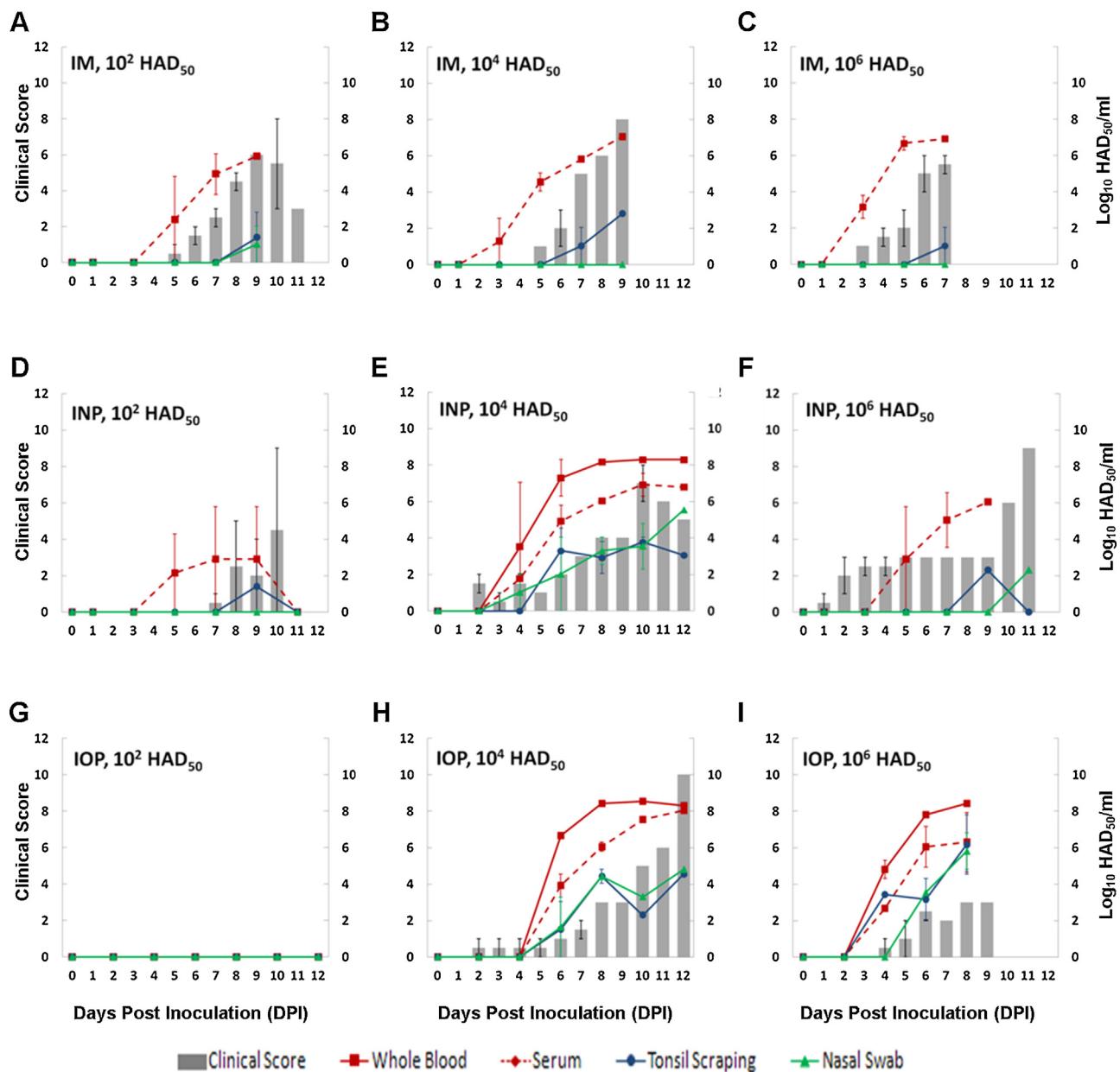


Fig. 2. Relationship between clinical score and virus shedding in pigs inoculated IM (A–C), INP (D–F), and IOP (G–I) with ASFV Malawi at 10^2 , 10^4 and 10^6 HAD₅₀. Lines indicating viral titer in whole blood, serum, tonsil swabs and nasal swabs are expressed on the right Y-axis. The daily clinical score (bars) are expressed on the left Y-axis.

3.2. Direct contact experiment

3.2.1. Inoculation and infectivity

For the IOP-inoculated donor pigs from the contact experiment, the overall incidence of ASFV infection was 66% (4/6 pigs), identical to the incidence in IOP-inoculated animals from the direct inoculation studies. Within DC groups, all animals, regardless of exposure time, were successfully infected (incidence = 100%) (Table 2).

3.2.2. Survival

All donor animals were euthanized between 7 and 9 dpi in accordance with the predetermined scheme to maintain 1:1 ratio with contact pigs. Naïve pigs were exposed to IOP-inoculated donors for 1, 2, or 3 days. Overall mean survival for all three exposure groups ($n=6$) was 10.3 ± 1.2 dpc with the longest onset of survival following onset of pyrexia (5.9 ± 2.0 days) compared to direct infection routes (Table 2, Fig. 1B). Animals exposed to donors for 3 dpc had the shortest overall survival compared to the 1 dpc and 2 dpc-exposure

groups. However, there were no statistical differences in the survival according to the Cox proportional analysis between different exposure durations.

3.2.3. Clinical signs

Among the IOP-inoculated donors, the mean number of days to onset of pyrexia was 3.6 ± 0.6 dpi. The mean duration to onset of clinical signs was 4.0 ± 1.7 dpi (Table 2). Two animals were pyrexic for 1–2 days but their temperature returned to within normal limits before euthanasia. These two pigs did not become viremic nor was shedding detected. One pig was not pyrexic by the time of euthanasia, but was viremic and shed virus nasally on the day of euthanasia (8 dpi). Four out of six pigs were obtunded with a mean onset of 4.0 ± 2.5 dpi. Donor animals did not have melena or hematochezia.

Amongst the DC-exposed pigs, onset of clinical signs began with pyrexia that was detected first within the 2 dpc-exposure group (3.5 ± 2.1 dpc, $n=2$) followed by the 1 dpc-exposure (4.5 ± 0.7 dpc, $n=2$) and 3 dpc-exposure (7.0 ± 0.0 dpc, $n=2$) groups. Obtundation

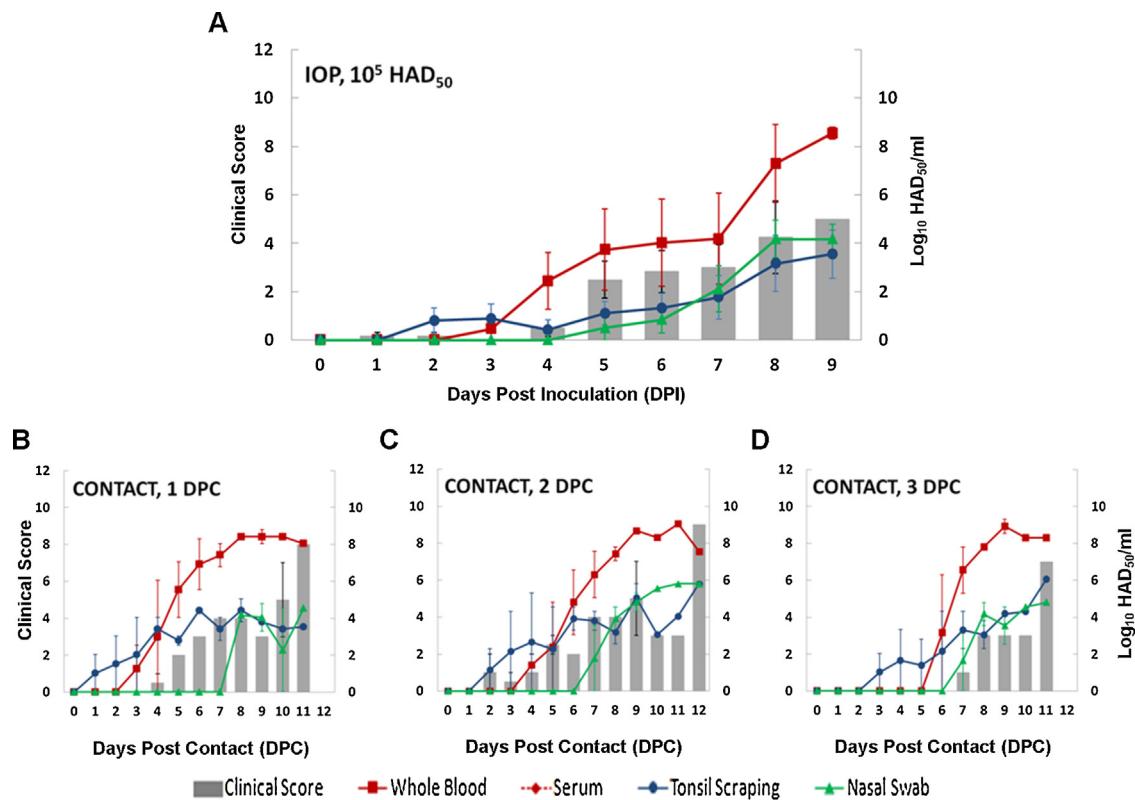


Fig. 3. Relationship between clinical score and virus shedding in donor pigs inoculated IOP (A) with ASFV Malawi at 10^5 HAD₅₀ and pigs exposed via Direct Contact (DC) for 24 h (B), 48 h (C) and 72 h (D). Lines indicating viral titer in whole blood, serum, tonsil swabs and nasal swabs are expressed on the right Y-axis. The daily clinical score (bars) are expressed on the left Y-axis.

was detected within 5 out of 6 pigs with a mean onset of 10.6 ± 1.1 dpc. Melena/hematochezia was detected in 2 of 6 pigs with a mean onset of 11.5 ± 0.7 dpc. The DC-exposed pigs had the highest overall maximum clinical score (6.8 ± 2.0); with the highest score in the 2 dpc group followed by the 1 dpc and 3 dpc groups.

3.2.4. Viremia and ASFV shedding

The mean detection of onset of viremia occurred within 4 out of 6 of the donor group pigs at 4.8 ± 2.2 dpi with the mean maximum whole blood titer of $7.4 \pm 3.2 \log_{10}$ HAD₅₀/mL. The onset of tonsil shedding was detected prior to viremia in 2 pigs. Onset of tonsil and nasal shedding was detected at 2.8 ± 1.5 and 6.5 ± 1.3 dpi respectively. Maximum titers for tonsil and nasal swabs were 4.1 ± 1.2 and $4.6 \pm 1.3 \log_{10}$ HAD₅₀/mL respectively (Fig. 3A, Table 3). The difference between onset of viremia and shedding averaged 1.3 ± 0.6 days.

Amongst contact-exposed pigs, mean onset of viremia was 5.2 ± 1.5 dpc and was detected first within the 1 dpc-exposure group (4.0 ± 1.4 dpc, n=2) followed by the 2 dpc and 3 dpc-exposure groups (5.0 ± 1.4 and 6.5 ± 0.7 dpc, n=2 per group). The overall mean highest whole blood titer was $8.8 \pm 0.3 \log_{10}$ HAD₅₀/mL, which was the highest amongst all routes (Table 3). Tonsillar detection of ASFV occurred prior to viremia in 4 pigs. The mean onset of tonsil and nasal shedding was detected at 3.5 ± 2.4 and 7.7 ± 0.6 dpc respectively, with mean maximum titers of 5.2 ± 0.8 and $4.7 \pm 0.1 \log_{10}$ HAD₅₀/mL. The difference between onset of viremia and shedding averaged 1.7 ± 1.7 days.

3.3. Postmortem lesions

All four routes produced comparable gross and histologic lesions in pigs at terminal stages of disease. The most consistent

lesion amongst pigs was enlarged and often hemorrhagic lymph nodes. The most severe lymphadenomegaly commonly involved the gastrohepatic lymph nodes. Splenomegaly ranged from mild to severe enlargement. Renal lesions varied from mild cortical petechiae to renomegaly with diffuse hemorrhage and congestion.

Histologically, lymphoid organs of all animals examined had perifollicular regions expanded by cellular debris, extensive hemorrhage, and distinct fragmented nuclear remnants, most consistent with lymphocyte apoptosis (Fig. 4A and B). Lymphoid follicles were still identifiable but less distinct compared to normal lymphoid architecture in naïve pigs.

3.4. Immunohistochemical localization of ASFV antigens

Examination of the distribution of virus and its association with specific cellular markers was performed on tissues collected from pigs of each inoculation route. Within lymphoid tissues of pigs with fulminant ASF, there was extensive cell-associated labeling with the anti-ASFV-VP30 antibody. Localization was predominately within interfollicular regions with few immunopositive cells within follicles (Fig. 4C). Within the tonsil of the soft palate, labeling was most prominent surrounding tonsillar crypts, with few labeled cells within the tonsillar crypt epithelium. This VP30-positive labeling did not colocalize with pancytokeratin.

Within all positive tissues, immunoreactive cells were morphologically consistent with large mononuclear cells (interpreted as macrophages). Combined multichannel immunofluorescence demonstrated that cells immunopositive for ASFV were also commonly positive for monocyte/macrophage cellular markers such as CD163 (Fig. 4D–G) and CD172a (not shown).

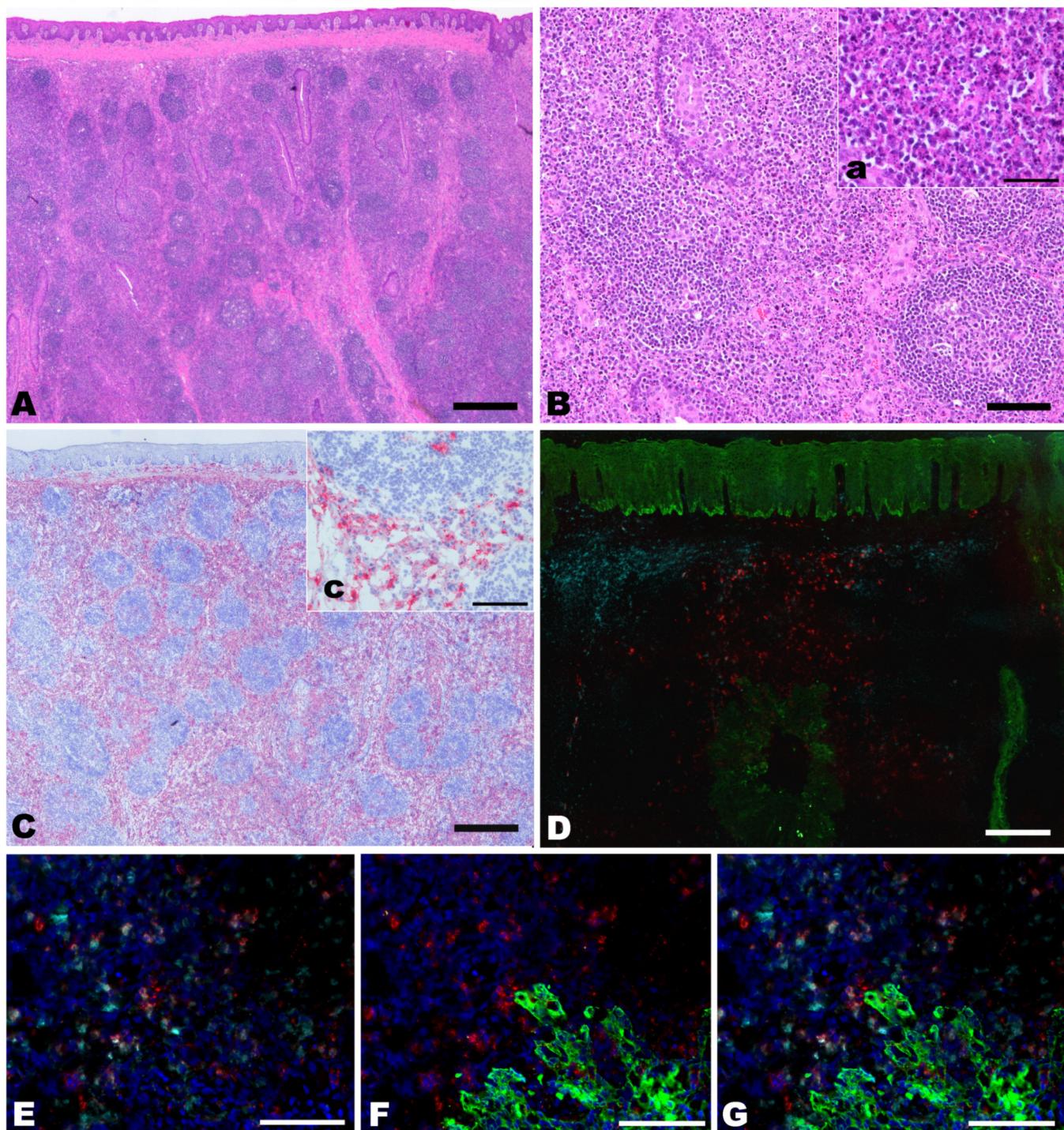


Fig. 4. Histopathology of lesions from pigs infected with ASFV-Malawi. Palatine tonsil, animal #38, INP ASFV-Malawi 10^2 HAD₅₀/mL; Lymphoid tissues including tonsil of the soft palate (A and B), lymph nodes, and spleen displayed loss of lymphocytes and karyorrhectic debris. Scale bars: (A) 500 μ m, (B) 100 μ m immunohistochemical staining (C) via Alkaline phosphatase polymer kit with anti-ASFV VP30 (1D9). Positive labeling extensively throughout interfollicular regions. Scale bar 500 μ m. Inset (c), region of interest at higher magnification. Multichannel immunofluorescence (D–G). (D) Low magnification three channel image containing anti-ASFV (red), pancytokeratin (green), and macrophage marker CD163 (aqua). Scale bar 500 μ m. (E) Three channel image containing Anti-ASFV (red), leukocyte marker macrophage marker CD163 (aqua) and nuclear staining with DAPI (blue). (F) Three channel image containing anti-ASFV (red), pancytokeratin (green), and nuclear staining with DAPI (blue). (G) Four channel merged image with colocalization of anti-ASFV (red) and leukocyte marker macrophage marker CD163 (aqua), pancytokeratin (green), and nuclear staining with DAPI (blue). Scale bar (E–G) 100 μ m.

4. Discussion

The goal of the investigation described herein was to evaluate dose- and route-dependent effects on the clinical course of ASF and viral dynamics of ASFV within domestic pigs. This work serves to

clarify and/or challenge previous conclusions derived from a collection of pathogenesis studies whose work is not directly comparable due to variability of virus strain, dose, inoculation technique, and detection methods. In addition, this work contributes to the establishment of challenge model systems for ASF which closely simulate

natural infection. The goals of the model systems were to achieve modes of challenging pigs that were (1) consistent with previous accounts of ASF, (2) consistent across experimental subjects, (3) compatible with the current understanding of natural routes of direct pig-to-pig transmission, (4) allowed control of dose and timing of challenge, and (5) provided natural engagement of host immune defenses. Recent studies have led toward standardization of clinical scoring systems for ASF ([de Carvalho Ferreira et al., 2012](#); [Galindo-Cardiel et al., 2013](#)), and the current work demonstrates adaptation of these systems where appropriate.

Four routes of challenge (IM, IOP, INP, DC) resulted in a similar clinical course of ASF that was consistent with previously described field cases ([Ayoade and Adeyemi, 2003](#); [Detray, 1963](#); [Sánchez Botija, 1982](#)) and experimental models ([Boulanger et al., 1967](#); [Colgrave et al., 1969](#); [Greig, 1972](#); [Greig and Plowright, 1970](#); [Heuschele, 1967](#); [McVicar, 1984](#); [Mebus et al., 1978](#); [Mebus and Dardiri, 1979, 1980](#); [Plowright et al., 1968](#); [Wilkinson and Donaldson, 1977](#); [Wilkinson et al., 1977, 1980](#)). Clinical signs did not vary substantially according to route of inoculation. The greatest consistency was achieved similarly by IM and DC challenge which both had 100% incidence of infection. However, these systems suffer from unnatural delivery of virus (IM) and inability to precisely control dose and timing of challenge (DC). All pigs challenged via DC became infected with ASFV regardless of length of exposure, thereby demonstrating that only 24 h of exposure to shedding animals is required for infection with ASFV. This finding is consistent with the previous works that demonstrated transmission after 24 h ([Greig and Plowright, 1970](#)) or 6 h ([Ekue et al., 1989](#)) of exposure to donor pigs. However, other studies have concluded that the quantity of virus shed 24 h following pyrexia was inadequate to ensure infection in pigs exposed via direct contact ([Heuschele, 1967](#); [Plowright et al., 1968](#)). INP inoculation had higher incidence whilst maintaining a natural exposure route and allowing control of dose and timing of challenge. Additionally, the shortest time to onset of clinical signs was observed within the INP groups, however this difference was not statistically significant.

Precise determination of pig infectious dose 50% ([PID₅₀], i.e. the minimum dose necessary to infected 50% of pigs) for the distinct inoculation routes was not possible due to logistical constraints which dictated some aspects of experimental design. However, the data herein suggests that the required dose of ASFV to establish infection is lowest for IM, intermediate for INP, and highest for IOP delivery of ASFV to domestic swine. Specifically, low dose inoculation (10²HAD₅₀) incidence proportions were 100% (IM), 50% (INP), and 0% (IOP). Yet, at 10⁴ HAD₅₀ all routes had 100% incidence of infection. This is consistent with previous studies that have demonstrated low ASFV IM dose (lower than 10⁵ TCID₅₀) ([Pan and Hess, 1984](#); [McVicar, 1984](#)) and high IOP dose (PID₅₀ higher than 10³ TCID₅₀) ([de Carvalho Ferreira et al., 2012](#); [Greig, 1972](#); [McVicar, 1984](#)). Additionally, Maurer et al. demonstrated that a minimal infectious dose of 10⁵ HAD₅₀ was necessary to infect pigs orally with ASFV ([Maurer and Griesemer, 1958](#)). The precise manner by which specific routes of inoculation of ASFV correlate with variable PID₅₀ via distinct pathogenesis mechanisms remains to be elucidated.

The route-specific clinical and virological characteristics described herein are likely to be the result of distinct route-determined, unique pathogenesis events in early ASFV infection. Pigs inoculated IM had the shortest mean survival duration; however the difference was not statistically significant. Shortest onset to viremia amongst IM-inoculated pigs was statistically significant. This may be due to more direct access to the vascular system associated with deposition within the muscle as compared to contact with intact mucosa as occurred with simulated natural challenge routes. Furthermore, the finding of several statistically significant

differences in the viremia and shedding parameters between the intramuscular and the direct contact route suggests that the intramuscular route is a poor simulator of natural infection. As a result, inoculation via IM should be avoided in experimental studies trying to mimic a natural course of disease. Contrastingly, both the IOP and INP inoculated pigs had comparable (i.e. not significantly different) results compared to the DC exposed pigs suggesting that IOP and INP are more likely to closely simulate natural infection.

The implied superior infectivity of ASFV via INP inoculation as compared to IOP was reflected in slightly higher incidence proportion and delayed onset of pyrexia and longer survival time seen among INP-inoculated pigs in comparison with all successfully IOP-inoculated pigs (including donors in DC experiment). However, IOP led to significantly higher shedding titers in the tonsil compared with INP inoculation. This effect may have been due to anatomic and physiological factors currently under investigation in our laboratory. IOP inoculation directly deposits the inoculum along the tonsil of the soft palate. While this tonsil has been documented as an early site of viral replication ([Heuschele, 1967](#); [Plowright et al., 1968](#)), to our knowledge, specific virus-host interactions in ASFV primary infection *in vivo* have never been explicitly described. Thus, the lower incidence of infection by the IOP route compared to INP-inoculation could be explained by lack of establishment of systemic disease subsequent to primary infection within the tonsil of the soft palate and other oropharyngeal tissues. Since low-dose INP-inoculated pigs had a higher incidence of successful infection than IOP, the nasopharynx may provide more permissive conditions for establishing systemic ASFV infection. INP inoculation targets nasopharyngeal tissues, including nasal tonsil, dorsal soft palate and walls/roof of the nasopharynx. The data presented in this study suggest that sites targeted by INP inoculation, or other tissues in the respiratory tract, may provide important portals for ASFV infection.

Increased inoculation dose of ASFV was associated with a significant decrease in survival duration of pigs. Animals inoculated with 10⁶ HAD₅₀, regardless of route, had a decreased mean number of days to onset of clinical signs (pyrexia, obtundation, hematchezia/melena) and survival compared to the mid and low dosage groups. The number of days to onset of viremia and shedding was decreased compared to mid and low doses as well, suggesting that a higher viral dose resulted in overwhelming primary replication and faster systemic dissemination of the virus. Paradoxically, the high-dose pigs had lower mean maximum clinical scores because of the rapidity of progression to death before the full severity of disease had occurred. Thus, maximum clinical score does not (in itself) describe the severity of disease but must be evaluated in context with duration of survival, virological characteristics, and pathologic findings.

During the clinical phase of infection, ASFV was variably detected in whole blood, serum and tonsil and nasal swabs. Amongst all challenge routes, viremia and shedding commonly either coincided with the onset of pyrexia or it occurred 2 days before or after pyrexia, which is in agreement with other studies ([Greig and Plowright, 1970](#); [McVicar, 1984](#)). The DC-exposed pigs had the highest mean ASFV titer within whole blood. Throughout the course of this study whole blood samples had titers of almost 2 log₁₀ higher than serum samples. This is consistent with previous studies that has described greater number of mature virus particles associated with erythrocytes and within circulating monocytes in whole blood, in comparison to free virions within serum ([Genovesi et al., 1988](#); [Wardley and Wilkinson, 1977](#)).

Detection of ASFV within secretions (tonsil scraping and nasal swabs) varied across infection routes and dosage groups. ASFV titers in tonsil scrapings and nasal swabs were generally quite similar, suggesting that virus was released from both regions and/or

virus quantities equilibrate between nasal and oral secretions. One noteworthy exception to this trend was amongst DC-exposed pigs wherein ASFV was consistently detected in tonsil scrapings (for 1–6 days) prior to detection in nasal swabs. Across all three inoculation routes, initial detection of ASFV shedding occurred at the same time, or subsequent to, virus detection in whole blood and serum. However within the contact challenge study, ASFV was detected in tonsil scraping samples 1–4 days prior to detection in blood of IOP-inoculated donors and DC-exposed pigs. This indicates that, under these distinct conditions, it was uniquely possible to detect local viral replication and shedding within the oral cavity prior to systemic dissemination. This is consistent with previous studies in which ASFV could be detected within oral and pharyngeal swabs 1–2 days prior to viremia and pyrexia (Greig and Plowright, 1970) or as early as 24 h post inoculation (hpi) in lymphoid and respiratory tract tissues (Heuschele, 1967; Plowright et al., 1968).

Gross and histologic changes in pigs in the late stages of disease were similar regardless of route of infection and were consistent with postmortem findings described in field and experimental cases of ASF. Systemic hemorrhages affecting the kidney and heart and pulmonary congestion and edema are similar to previously described lesions (Detray, 1963; Mebus, 1988). Amongst individual pigs, severity of lesions varied from mild to severe based upon recently published recommendations for standardization of pathologic findings for ASF (Galindo-Cardiel et al., 2013). The hallmark depletion and apoptosis of lymphocytes throughout the lymphoid tissue with subsequent infiltration of macrophages commonly described for ASF (Gómez-Villamandos et al., 2003) was observed histologically within this study. Detailed pathological description will be forthcoming in a separate manuscript (Howey et al., in preparation).

Immunohistochemical visualization of ASFV antigen was demonstrated in tonsil of the soft palate of pigs in terminal stages of ASF, further confirming the presence and characterizing the distribution of virus within tissues. Immunoreactive cells were large mononuclear cells and colocalization with monocyte/macrophage cellular antigen suggested that the predominate infected cell type is of monocytic origin. Colocalization of ASFV with CD163 supports previous suggestions that this molecule may serve as an important ASFV receptor (Alonso et al., 2013; Sánchez-Torres et al., 2003). Additionally, this is consistent with numerous reports indicating moncytotropicism of ASFV (Detray, 1963; Tulman et al., 2009; Wardley and Wilkinson, 1978).

5. Conclusions

The current study has demonstrated that four modes of challenging pigs with ASFV result in similar ASF syndromes with slight variations. The IM route may be advantageous for the study of late stage ASF events; however several significant differences from DC indicated this is not an optimal system to simulate natural infection. DC would be preferable when simulating a natural manner of transmission was utmost priority; however this system is expensive due to the inclusion of additional (donor) animals in each experiment and does not allow control of dose quantity and timing. The two simulated natural manners of inoculation, IOP and INP, allowed precise control of timing and dose of ASFV delivery and had similar incidence of successful infection of pigs. Overall, the slightly lower minimum infectious dose and slightly higher incidence of infection subsequent to INP inoculation in these experiments suggest that INP may be the preferred route of inoculation for pathogenesis and vaccine challenge studies of pigs with ASFV. Further validation of these techniques with larger sample sizes would contribute to stronger conclusions regarding the differences between the infection systems described herein.

Conflict of interests

The authors do not have any financial or personal conflict of interests.

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EH planned and carried out the live animal experiments, virus isolation, immunomicroscopy and drafted the manuscript. VO provided expertise in conceptual design, as well as optimization and troubleshooting of techniques. HF performed the statistical analysis and helped with manuscript revisions. MB consulted and contributed on all aspects of study design and implementation; also provided critical review of manuscript. JA conceived the study, oversaw the implementation of experiments, helped draft and revised the manuscript. All authors read and approved the final manuscript.

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